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ARTIFICIAL HYBRIDIZATION AND *IN VITRO* SEED GERMINATION IN *PHALAENOPSIS* SP.

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Abstract

Phalaenopsis (moth orchid) is one of the most popular indoor orchids, being cultivated for their long-lasting flowers that are produced all year round. A modern approach for inducing variability and creating new marketable cultivars employs artificial hybridization followed by in vitro seed germination. For these purposes, twelve cultivars selected for their highly variable morphology were used in six direct and reciprocal hybrid combinations. The hybridizations were performed in January, because the percentage of pollination and capsule formation are higher compared to hybridizations performed during summer. Capsules were obtained in eight hybrid combinations and were harvested before dehiscence. Seed germination was tested both on MS basal medium devoid of plant growth regulators and on a MS variant supplemented with 1.5 mg/l BAP. The first variant provided the best rate of proliferating embryos (70-90%), for the next hybrid combinations (1x2, 12x18, 16x11). The other two hybrid combinations (3x4, 14x13) that produced capsules showed no germination. For plantlet formation, protocorms were transferred on a MS medium supplemented with 6.0 mg/l BAP. Plantlets were successfully obtained from seeds after 30-35 days.

Key words*: hybridization, in vitro, seed germination, Phalaenopsis, protocorms.*

INTRODUCTION

Moth orchids belong to genera *Phalaenopsis* and *Doritaenopsis* (intergeneric hybrids between *Dortis* and *Phalaenopsis*). The first genus comprises various types of petal sizes, shapes and colours (Park *et al.*, 2002), making it very important for the potted plant industry. In our country, orchids come exclusively from import. For this reason, we evaluated the potential of marketed varieties for obtaining new cultivars, and also tested *in vitro* germination of hybrid seeds.

MATERIALS AND METHODS

Artificial pollination

The biological material was represented by 12 commercial varieties of *Phalaenopsis*, which were selected from the local market, based on their morphological characteristics, such as flower colour and size.

In order to obtain a high number of hybrid capsules, the artificial pollination was performed during winter time (January), because the level of capsule formation is higher in this season (Balilashaki *et al.*, 2015).

A total of 60 flowers belonging to the 12 commercial varieties were employed in direct and reciprocal crossing system (Table 2).

The artificial pollination was performed in the first days after flowers opened, by detaching the anther cap and pollinia using a toothpick and placing the pollinia over the stigma of the female genitor (which was previously emasculated).

Five flowers were used for each hybrid combination, which was labelled with the genitor plant numbers.

Capsules harvesting

The 60 cross-pollinated flowers produced a total of 31 capsules.

These were harvested after five months from artificial hybridization (Figure 1-a), before opening.

Harvested pods were kept in self-sealing plastic bags for preventing dehydration.

Table 1. Commercial varieties characteristics

No.	Plant label	Flower colour	Flower size
1	1	white	small
2	2	white	large
3	3	light violet with dark violet spots	large
4	4	green-yellow with spots	small
5	5	lavender	large
6	6	orange	middle
7	11	white	large
8	12	light pink	large
9	13	yellow-green	large
10	14	magenta	middle
11	16	green-yellow with pink dots	middle
12	18	dark purple	large

***In vitro* culture**

Sterilization of the unopened capsules was initially carried out by immersion in 70% ethanol, for 3 minutes. The second step consisted in treating the capsules with a 50% sodium hypochlorite (NaOCl) solution containing 2-3 drops of Tween, for 15 minutes, with continuous stirring. After sterilization, the capsules were washed three times with sterile distilled water. The seeds were recovered by vertically cutting the capsules with a scalpel and were immediately distributed on nutrient culture media in Petri dishes (Figure 1- b, c, d).

A Murashige and Skoog (1962) (MS) culture medium without growth hormones (M1) and a MS supplemented with 1.5 mg/L 6-benzylaminopurine (BAP) (M2) were used for inoculation. Both media contained 30 g/l sucrose and 6.5 g/l agar for solidification. Before autoclaving, the pH of the media was adjusted to 5.7 using 0.1 N NaOH or 0.1 N HCl. Autoclaving was performed at 121 °C and 1 atm, for 20 minutes.

The cultures were maintained in the growth chamber at a temperature of 25 ± 10 °C, with a photoperiod of 16 h and intensity of 2500 lux, provided by cold, white, fluorescent tubes.

Subcultivation was performed at 12 weeks after seed germination by cutting the protocorms that resulted from the seeds into pieces and placing them on MS culture medium supplemented with 6 mg/L BAP (Figure 1-e).

Two fresh media variants were employed for the development and *in vitro* rooting of regenerated protocorms: MS supplemented with 6.0 mg/L BAP, and ½ MS supplemented with 6.0 mg/L BAP. Both variants were also supplemented with 2% activated charcoal.

Observations were made on the percentage of explant contamination, explant color, duration of protocorm formation and seed germination percentage.

Table 2. The hybrid combinations and capsule formation (D = Direct crossing; R = Reciprocal crossing)

Hybrid combination (♀ x ♂)	Type of crossing	Flower size	No. of flowers	No. of capsules
1 x 2	D	small x large	5	5
2 x 1	R	large x small	5	1
3 x 4	D	large x small	5	3
4 x 3	R	small x large	5	4
5 x 6	D	large x middle	5	-
6 x 5	R	middle x large	5	4
11 x 16	D	large x middle	5	-
16 x 11	R	middle x large	5	5
13 x 14	D	large x middle	5	-
14 x 13	R	middle x large	5	4
12 x 18	D	large x large	5	5
18 x 12	R	large x large	5	-

Statistical analyses

The data interpretation was made using the analysis of variance (ANOVA) and the differences between plants and culture media were performed using Tukey's test ($P \leq 0.05$).

RESULTS AND DISCUSSIONS

As shown in table 2, eight out of 12 hybrid combinations produced 31 capsules, while the other four did not produce any capsules. A possible explanation for these results could be the use of plant with small flowers as male genitors, which, according to Hicks (2000), may not develop pollen tubes long enough to fertilize the egg cell of large flowers. Only two (3 x 4, 14 x 13) of the hybrid combinations that produced capsules, did not germinate on any type of culture media.

The beginning of germination was marked by the swelling and growing of embryos.

To produce plants from seeds, the orchid embryos have to develop protocorm-like bodies in the first stage, and then plantlets. Protocorm formation was observed ten days after inoculation and after 30 days there was an increase in chlorophyll content and the formation of first leaves and rhizoids began.

The protocorms with green rhizoids and first leafs were passed on fresh MS medium,

supplemented with 6 mg/L BAP, after about 80 days.

It is well known that many orchid species germinate well in less complex environments, *i.e.* with fewer nutrients, which are not necessary for further growth and development (Rasmussen, 1995).

However, in our case, cytokinin enriched medium ensured a good development of protocorms, most of which forming leafs and roots approximately two months after inoculation. In the next two weeks some of the plants formed two leaves and 1-2 roots (Figure 1-f).

The development of *Phalaenopsis* seedlings *in vitro* could take 50 to 724 days and another 4.2 to 31.5 months for further development (Arditti, 1992).

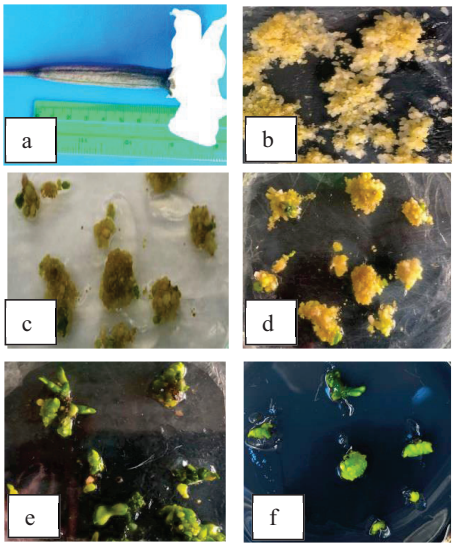

Figure 1. Capsule formation and asymbiotic seed germination of *Phalaenopsis* cultivars. **a** = hybrid capsule; **b, c, d** = seed germination on MS media - protocorms formation; **e, f** = protocorms, rhizoids and first leaves on MS media supplemented with 6.0 mg/L BAP and activated charcoal

The best protocorm development was recorded for the following combinations: 1 x 2, 16 x 11 and 12 x 18. In two of the combinations (3 x 4 and 14 x 13), seed germination was not achieved, while in three variants (2 x 1, 4 x 3 and 6 x 5) only slight germination was observed.

Poor seed germination may be attributed to insufficient maturation, which results in a much slower germination process, or complete lack of germination.

In vitro culture techniques use green capsules with immature seeds.

The seeds in the green capsules have a higher germination rate. In our study, mature seeds, brown or white in color and recovered from mature, brown capsules, did not germinate on any media variant. The combinations 2 x 1 and 3 x 4 had mature capsules with brown seeds, while variants 6 x 5, 14 x 13 and 4 x 3 had white seeds (Table 3).

Table 3. Colour of seeds used for inoculation

Hybrid combination	Green	Yellow	Brown	White
1x 2	x	x		
2 x 1	-	-	x	-
3 x 4	-	-	x	-
4 x 3	-	-	-	x
6 x 5	-	-	x	x
16 x 11	x	-	x	-
14 x 13	-	-	-	x
12 x 18	x	-	x	-

Under natural conditions, it is difficult for *Phalenopsis* species to produce seeds, and it takes a long period of time for these to germinate. *In vitro* however, *Phalaenopsis* orchids can be grown more easily and with a high rate of propagation. Knudson (1922) has shown that orchid seeds are able to germinate in laboratory conditions without the presence of the fungus. Knudson (1922) has shown that the fungus is converting the starch into the medium in sugar and this sugar has been used for germination. He replaced starch with sugar and orchids grow very well without the fungus (Thomas, 1992).

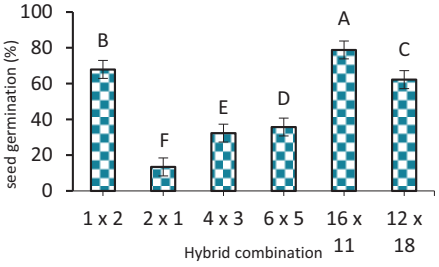

Figure 2. Hybrid combination effect on seed germination and protocorms formation. Mean values with the same letter are not significantly different at $P \leq 0.05$.

Three (16 x 11, 1 x 2 and 12 x 18) of the six hybrid combinations averaged higher than 50%

in protocorm formation, the best being 16 x 11 with 78.8% (Figure 2). The lowest results were recorded for combination 2 x 1, in which the plant with the smallest flowers (no. 1) was used as male genitor.

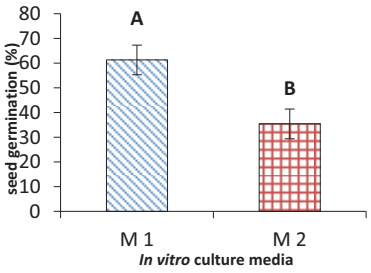

Figure 3. The influence of *in vitro* culture media on seed germination and protocorm formation. Mean values with the same letter are not significantly different at $P \leq 0.05$.

There are statistically significant differences between culture media, the variant without growth hormones producing a higher percentage of protocorms compared to the variant supplemented with 1.5 mg/L BAP (Figure 3).

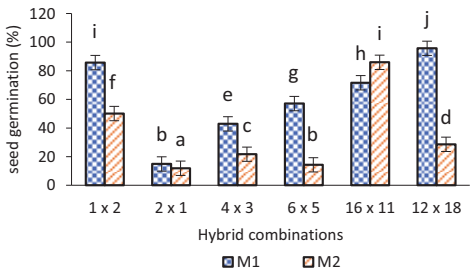

Figure 4. The effect of hybrid combination and culture media interaction on seed germination and protocorms formation (M1 = culture medium without growth hormones, M2 = culture media supplemented with 1.5 mg/L BAP). Mean values with the same letter are not significantly different at $P \leq 0.05$.

The best protocorm formation was recorded on the media variant without hormones for five out of the six hybrid combinations, with values reaching more than 70% in three cases: 1 x 2 (85.7%), 16 x 11 (71.6%) and 12 x 18 (95.7%). The second media variant (with added hormones) produced better results only in the case of one (16 x 11) of the six combinations (Figure 4).

CONCLUSIONS

The media without growth hormones provided the best rate of embryo proliferation (70-90%), for hybrid combinations: 1 x 2, 12 x 18 and 16 x 11.

The use of plants with small flowers as male parent may impair artificial hybridization.

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